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Pathogen resistance and adaptation to heat stress

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16.1 Introduction

Heat treatment of foods is one of the oldest and most effective physical processing strategies for controlling foodborne pathogenic microorganisms. The effectiveness of heat in inactivating the microorganism of concern is dependent upon the severity of the treatment and the ability of the pathogen to withstand the treatment, recover from injury, proliferate, and withstand subsequent treatments that may occur. Effective injury repair can result in removal of damaged DNA and proteins followed by resynthesis of cellular constituents and repair of cellular membranes. Although injured microorganisms may be more susceptible to inactivation, they may also utilize mechanisms for increasing resistance to subsequent stresses. It is important that heat treatments ensure lethality without enabling enhanced resilience or cross-protection following inadequate inactivation attempts. Another consideration is the avoidance of excessive damage to the food product. Clearly, there is a narrow range of time/temperature treatments that can be applied to deliver maximal pathogen inactivation and minimal food product adulteration.

There needs to be assurance that foodborne pathogens are killed during heating. Under certain conditions of food processing, microorganisms could become more heat resistant. Prior exposure to low heat may also render the organism more resistant to a subsequent heat treatment that would otherwise be lethal (Murano

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and Pierson, 1992; Lou and Yousef, 1996; Juneja *et al.*, 1997). Heat shock proteins are synthesized concurrent with increased heat resistance. It is possible that heat shock proteins protect cells either by preventing the denaturation of normal proteins, or through the removal of heat-damaged proteins (Nguyen *et al.*, 1989). 'Heat-shocked' cells require greater inactivation temperatures than 'non-heat-shocked cells' to achieve the same level of lethality (Farber and Brown, 1990).

The induced thermotolerance of microorganisms from heat shock is of concern to food processors that typically use temperatures below 65 °C to process their products. Other conditions where the increased heat resistance of microorganisms could be problematic include products left on warming trays before final heating or a process failure. The additional heat adaptation of pathogens needs consideration to ensure required inactivation of microorganisms, leading to safer foods.

16.2 Predicting pathogen resistance

The heating times at a specific temperature are often increased to ensure the inactivation of high microbial populations in foods. Incorporated into this design is the normal expected microbial concentration for a given product. The heat resistance of bacteria has been historically described by two characteristic parameters, D- and z-values. In principle, the D-value is the time necessary to inactivate 90 % of the initial population of microorganisms present in a food at a specific temperature, whereas the z-value describes the temperature change necessary to result in a 90 % change in the D-value. Although universally accepted, these calculations deviate from the expected linear relationships for microorganisms fairly regularly, and especially for bacterial spores.

It has long been accepted that when bacteria are killed by heat they die at a constant rate, i.e. via first-order kinetics (Stumbo, 1973; Tomlins and Ordal, 1976). This model of thermal inactivation forms the basis of calculations used in thermal processing and has served the food industry and regulatory agencies for decades. Unfortunately not all the microbial cells in a given population have identical heat exposure or resistance, and the improbable chance of a heat-sensitive target in one cell determining the death rate of the entire cell population fails (McKee and Gould, 1988). There have been significant deviations from predicted relationships observed by many researchers using different methodologies (Tomlins and Ordal, 1976; Pflug and Holcomb, 1983; Cole *et al.*, 1993). Frequently a shoulder or lag period is observed when bacterial populations remain constant or a tailing slower death rate is observed when subpopulations of more resistant bacteria are present (Fig. 16.1).

At present, there is no satisfactory explanation for the variability in thermal death kinetics. Some investigators have suggested that deviations from linear survival curves result from heterogeneous cell populations (Hansen and Riemann, 1963). Possible explanations for the 'shoulder effect' include poor heat transfer and the requirement for sufficient cellular injury before observed cell death leads to the expected first-order inactivation relationship. Other theories concentrate on

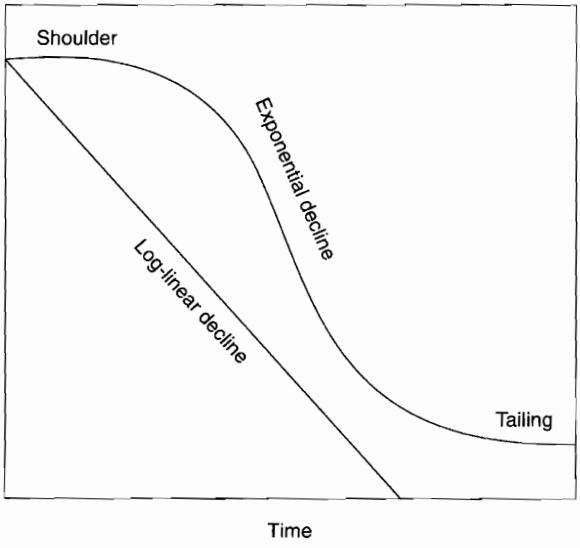

Fig. 16.1 Thermal inactivation of microorganisms. The straight line represents the traditional first-order kinetics of log number of survivors declining in a linear manner with time. The sigmoidal curve depicts a more realistic representation.

the need for multiple inactivation events or the activation of spores to germinate making them more susceptible to the lethal effects of heat. Clumping of small numbers of cells or spores may protect cells against thermal destruction (Stumbo, 1973; Hansen and Riemann, 1963). In response to environmental conditions or even physiological changes during its life cycle, an individual cell can have varying degrees of heat resistance (Cerf, 1977). Also, heat resistance can be acquired, as a result of sublethal heating, and lead to deviations from linearity in plotted survival curves. There have been numerous attempts to explain these deviations from the expected linear survival survivor curves (Casolari, 1988; Gould, 1989; Pflug, 1990; Whiting, 1995).

16.3 Factors influencing the development of resistance

An appropriate heat treatment designed to achieve a specified lethality of microorganisms is influenced by many factors, some of which can be attributed to the inherent resistance of microorganisms, while others are due to environmental influences. Examples of inherent resistance include the differences between species and the different strains or isolates of bacteria (assessed individually or as a mixture) and the differences between spores and vegetative cells. Environmental factors include those affecting the microorganisms during growth and formation of cells or spores (i.e. stage of growth, growth temperature, growth medium, previous exposure to stress) and those active during the heating of bacterial suspensions,

such as the composition of the heating menstruum, water activity (a_w), pH, added preservatives, method of heating, and methodology used for recovery of survivors.

The persistence of heat shock-induced thermotolerance appears to be a function of many factors including the heat shock temperature, the previous incubation temperature of the cell, and the metabolic state of the cell (Lindquist, 1986). Temperature fluctuations are a common occurrence in food processing environments, as well as during transportation, distribution, and storage or handling of foods in supermarkets or by consumers. Therefore, temperature can play a role in the food environment stresses to which bacterial cells will be exposed. Guidelines have been suggested to avoid environmental stresses so as not to enhance pathogen survival during thermal processing procedures (Juneja *et al.*, 1997).

There are time–temperature combinations that produce maximum thermotolerance following heat shock. Temperatures between 45 ° and 50 °C are optimal for development of the heat shock response in mesophilic bacteria (Lindquist, 1986). In one study, *Listeria monocytogenes* cells in sausage required 120 min at 48 °C prior to final treatment at 64 °C to exhibit a 2.4-fold increase in thermal resistance as compared to non-heat-shocked cells (Farber and Brown, 1990). Lower exposure times reduced the heat resistance effect. The authors also reported that the heat-shocked cells retained their increased heat resistance for 24 h after storage at 4 °C.

Mackey and Derrick (1986) increased the heat resistance of *Salmonella* Typhimurium (*Salmonella enterica* serovar Typhimurium) to a range of lethal temperatures (52–59 °C) in tryptone soya broth by prior exposure of the cultures to sublethal heat shock at 48 °C for 30 min. Greatest heat resistance was reached within 30 min of exposure and persisted for 10 h. A similar effect was demonstrated with *S.* Thompson when the organism was preheated at 48 °C and then subjected to 54 or 60 °C in tryptone soya broth, liquid whole egg, 10 % (w/v) or 40 % (w/v) reconstituted dried milk, or minced beef (Mackey and Derrick, 1987).

Shenoy and Murano (1996) heat shocked *Yersinia enterocolitica* in brain–heart infusion broth at 45 °C for 60 min and subsequently observed an increased number of survivors at 55 or 60 °C when compared with non-heat-shocked cells. The thermotolerance of *L. monocytogenes* at 65 °C increased with the duration of the heat shock for up to 120 min, regardless of the heat shock temperature from 40 to 46 °C (Pagan *et al.*, 1997).

In contrast to these studies demonstrating a parallel increase in heat resistance with the increase in the time of heat shocking, Murano and Pierson (1992) heat-shocked *Escherichia coli* O157:H7 cells in trypticase soy broth (TSB) at 30, 34, 42, or 45 °C for 0, 5, 10, or 15 min and reported that heat shocking at 42 °C for 5 min resulted in the greatest log number of survivors at 55 °C compared with non-heat shocked controls. Linton *et al.* (1990) heat-shocked log phase cells of *L. monocytogenes* Scott A in TSB supplemented with 0.6 % yeast extract (TSYE) at 40, 44, and 48 °C for 3, 10, and 20 min, followed by heating at 55 °C for 50 min. The optimum heat shock condition for increasing subsequent heat resistance was 48 °C for 10 min where heat resistance at 55 °C increased 2.3-fold in non-selective agar (TSYE) and 1.6-fold in selective agar (McBride *Listeria*). Cells that were

heat-shocked at 48 °C for 10 min were consistently more resistant to subsequent heating at 50, 55, 60, and 65 °C than non-heat-shocked cells.

Juneja *et al.* (1997) used a submerged-coil heating apparatus to determine the effect of prior heat on *E. coli* O157:H7, inoculated in a model beef gravy, and the persistence of the thermotolerance at 4, 15, and 28 °C after heat shock. When beef gravy samples inoculated with a four strain cocktail of *E. coli* O157:H7 were subjected to sublethal heating at 46 °C for 15–30 min, followed by cooking to a final internal temperature of 60 °C, the organism survived 1.56-fold longer than non-heat-shocked cells. In this study, a linear decline in the log number of survivors with time was observed. The induction of thermotolerance by heat shock was maintained for at least 48 h at 4, 15, or 28 °C. However, when a similar study was conducted in bags of ground beef, heated using a water bath, the primary thermotolerant response of *E. coli* O157:H7 switched to non-linear inactivation kinetics, resulting in the presence of a shoulder (Juneja *et al.*, 1997). Unlike the beef gravy, it was interesting to note that *E. coli* O157:H7 cells in beef lost their thermotolerance after 14 h at 4 °C and after 24 h in beef held at 15 or 28 °C.

Listeria monocytogenes suspended in tryptic phosphate broth (TPB), heat-shocked at 46 °C for 30 min, and held at 4, 10, or 30 °C before heating at 58 °C, resulted in thermotolerance being maintained for a longer time at 4 and 10 °C compared with cells stored at 30 °C (Jorgensen *et al.*, 1996). Even after 48 h, cells grown and held at 4 °C after the heat shock were two-fold more heat resistant than non-heat-shocked cells grown at 4 °C. These findings have important implications for the survival of pathogens in pre-cooked foods which are then stored at refrigeration temperatures.

Bunning *et al.* (1990) heat-shocked stationary phase cells of *L. monocytogenes* grown at 35 °C (control), at 42, 48, and 52 °C for 5–60 min prior to heating at 57.8 °C. Although heat shocking at 42–48 °C for 5–60 min consistently increased *D*-values at 57.8 °C by 1.1 to 1.4-fold, these data were not statistically different from non-heat-shocked cells. When similar experiments were conducted with *Salmonella* Typhimurium, *D*-values increased by 1.1 to 3.0-fold and were significantly different than non-heat-shocked cells. When *L. monocytogenes* cells were held at 42 °C, thermotolerance remained at a maximum level for at least 4 h. However, in preheated cells incubated at 35 °C, the increased thermal tolerance lasted less than 1 h.

A given atmosphere combined with heat stress can further increase the heat resistance of *E. coli* O157:H7. In a study by Murano and Pierson (1992), when log phase cells of *E. coli* O157:H7 grown in TSB at 30 °C were subjected to heat shock at 42 °C for 5, 10 or 15 min before final heating at 55 °C, *D*-values increased by more than 2-fold for aerobically grown cells, and 1.5-fold when grown under anaerobic conditions. The *D*-values of anaerobically grown non-heat-shocked controls at 55 °C, were significantly higher than those of aerobically grown controls. Anaerobiosis is considered a form of stress to bacterial cells.

Using a submerged coil heating apparatus set at 58 °C, *L. monocytogenes* cells, grown at either 10 or 30 °C, were shown to have no difference in thermotolerance, but were significantly ($p < 0.001$) more heat resistant (1.5-fold) than cells grown

at 4 °C (Jorgensen *et al.*, 1996). The heat shock-induced thermotolerance could be lost following a given growth period as a result of metabolic turnover. For example, cells grown at 4, 10 or 30 °C showed the same amount of reduction when held at 30 °C as a result of new protein synthesis and degradation following heat shock.

The degree to which *E. coli* O157:H7 heat-shocked and non-heat-shocked cells are injured following a heating process and the ability of injured cells to repair themselves under aerobic and anaerobic conditions has been described by Murano and Pierson (1993). Bacteria encounter stress from both oxygen surplus and deprivation (Potter *et al.*, 2000). Not surprisingly, the *D*-values of heat-shocked cells increased along with the numbers of injured cells as a result of heat shock (Murano and Pierson, 1993). When cells were recovered under anaerobic conditions; a higher recovery of injured cells was observed along with higher *D*-values as compared with cells recovered aerobically. This phenomenon was observed regardless of whether the cells were previously heat-shocked. A possible explanation includes the spontaneous formation of toxic oxygen radicals in aerobic media, which heated cells are unable to deactivate due to the heat inactivation of detoxifying enzymes such as catalase and superoxide dismutase (SOD). Since anaerobic storage is a practice that is prevalent in the food industry for shelf-life extension of processed meats, the microbiological safety of such foods should be of concern because of the enhanced recovery of injured pathogens following heat treatment.

Linton *et al.* (1992) assessed the effect of recovery medium on the survival of heat-injured *L. monocytogenes*. The *D*-values at 55 °C for heat-shocked (48 °C for 10 min) log phase cells of *L. monocytogenes* Scott A were 2.1-fold higher than non-heat-shocked cells on non-selective agar (TSYE) incubated aerobically and similarly 2.2-fold higher for cells enumerated anaerobically on TSYE agar. On selective medium (McBride *Listeria*-ML), the values were 1.4-fold higher than those of non-heat-shocked cells. Interestingly, no growth was observed on ML agar incubated anaerobically. Fedio and Jackson (1989) exposed stationary-phase cells of *L. monocytogenes* Scott A to a preheating treatment of 48 °C for 1 h in TSYE broth followed by heating at 60 °C for 20 min. Preheating rendered the pathogen more resistant, and a 4-$\log_{10}$ higher number of cells were recovered as compared to non-heat-shocked cells regardless of the recovery medium (selective or non-selective).

Increases in *D*-values (up to 22 % compared to the control) for *Salmonella* Enteritidis (*Salmonella enterica* serovar Enteritidis) following heat shock (42 °C for 60 min) were reported by Xavier and Ingham (1997). This study suggested that: (i) short-term temperature abuse of foods containing *S.* Enteritidis may render the cells more resistant to subsequent heat treatments; (ii) anaerobic microenvironments may enhance survival of heat-stressed cells (i.e. increases in *D*-values up to 28 % compared with the aerobic value); and (iii) heat shock results in the over-expression of proteins that may be related to increased thermotolerance.

Heat stress conditions may be encountered in minimally processed, cook–chill processed foods of extended durability. Slow heating rate/long come-up times and low heating temperatures employed in the production of *sous-vide* cooked foods

expose microbial cells to conditions similar to heat shock. Stephens *et al.* (1994) and Kim and Thayer (1996) have shown that slowly raising the cooking temperature enhanced the heat resistance of *L. monocytogenes* in broth and pork, respectively. Hansen and Knochel (1996) reported a difference between slow (0.3–0.6 °C/min) and rapid (>10 °C/min) heating and the heat resistance of *L. monocytogenes* in cooked beef at a pH of 6.2, but not less than pH 5.8.

Tsuchido *et al.* (1974, 1982) observed increased thermotolerance of *E. coli* by raising the temperature of the cell suspension from 0 to 50 °C at various rates prior to holding at 50 °C. Also, Thompson *et al.* (1979) increased the thermotolerance of *S.* Typhimurium in beef under realistic conditions of constantly rising temperature. Subsequently, Mackey and Derrick (1987) reported that the heat resistance of *S.* Typhimurium, measured as survival following a final heating at 55 °C for 25 min, increased progressively as cells were heated during linearly rising temperatures. In that study, cells were heated at a rate of 0.6 or 10 °C/min from 20 to 55 °C, and then subjected to a heat challenge at 55 °C for 25 min. The authors reported that the extent of induced thermotolerance was inversely related to the rate of heating, i.e. the slower the temperature rise, the greater the increase in resistance. Quintavala and Campanini (1991) determined the heat resistance of *L. monocytogenes* 5S heated at 60, 63, and 66 °C in a meat emulsion at a rate of 5 °C/min resulted in *D*-values at least two-fold higher than cells of *L. monocytogenes* in meat, exposed to instantaneous heating.

Cellular targets for heat damage are ribosomes, nucleic acids, enzymes and/or proteins (Abee and Wouters, 1999). Mild heat treatment can also lead to modifications of the cell membrane by increasing the saturation and length of the fatty acids needed to maintain optimal fluidity of the membrane and activity of intrinsic proteins (Russell and Fukanaga, 1990; Russell *et al.*, 1995). In a study on the physiological state of cell membranes from Gram-negative bacteria, the total saturated fatty acids (SFA) and total unsaturated fatty acids (UFA) were highly influenced by temperature (Dubois-Brissonnet *et al.*, 2000). When the temperature was increased from 15 to 40 °C, SFA increased from 25 to 39 %, whereas UFA decreased from 66.5 to 51 % (Dubois-Brissonnet *et al.*, 2000).

Stress-adapted bacteria are capable of resisting similar (homologous) or different (heterologous) stresses. Termed 'cross-protection', exposure to one stress is capable of altering resistance to another stress and is mediated by the *rpoS* gene. Wang and Doyle (1998) reported that sublethal heat treatment of *E. coli* O157:H7 cells substantially increased their tolerance to acidity. In contrast, although heat resistance increased in bacterial spores produced at higher temperatures (Condon *et al.*, 1992; Sedlak *et al.*, 1993), lactic acid through a lowering of pH was effective in reducing the heat resistance of the spores produced at higher temperatures (Palop *et al.*, 1996). Lou and Yousef (1997) examined the effect of sublethal heat (45 °C for 1 h) on the resistance of exponential phase cultures of *L. monocytogenes* to certain environmental stresses and found that this greatly increased resistance of the pathogen to normally lethal doses of hydrogen peroxide, ethanol and NaCl. As a consequence of stress-induced cross-protection, Lou and Yousef (1997) concluded that certain stresses might counterbalance the benefits of multiple stress

hurdles. In *L. monocytogenes*, stress induced by heat treatment did not lead to acid tolerance, but cells induced by low pH did become more resistant to heat, salt concentration and antimicrobial peptides (Hill and Gahan, 2000).

Different cross-protection adaptations of specific pathogens must be taken into account when assessing the microbial safety of minimal food processing technologies alone or in combination. Komatsu *et al.* (1990) showed that exposure of yeast cells to a heat shock conferred protection against freezing in liquid nitrogen. Additionally, it was found that carbon starvation in *E. coli* elicited an essential need for DnaK expression in order to acquire heat and oxidation resistance (Rockabrand *et al.*, 1998). Further research is needed to fully understand the levels and types of stresses necessary to elicit universal or specific adaptations among foodborne pathogens. In response to environmental conditions or even physiological changes during its life cycle, an individual cell can have varying degrees of heat resistance (Cerf, 1977). The heat resistance can be acquired as a result of sublethal heating and lead to deviations from linearity in plotted survival curves. There have been numerous attempts to explain these deviations from the expected linear survivor curves (Casolari, 1988; Gould, 1989; Pflug, 1990; Whiting, 1995).

16.4 Targets of heat damage

Heat is believed to be uniformly distributed in a cell, resulting in damage to only the most sensitive molecules within that cell (Moats, 1971). Potential targets of heat damage have been implicated with associations to various pathogen viabilities. These include proteins, enzymes and cellular membranes, as well as nucleic acids (Marquis *et al.*, 1994).

Even mild heat is known to play a key role in DNA damage and hydrolysis. Purine bases were first shown to be liberated from DNA at elevated temperatures (80–95 °C) followed by depyrimidation of cytosine and thymine bases at approximately 5 % the rate of depurination (Lindahl and Nyberg, 1972; Lindahl and Karlstrom, 1973). Apurinic sites led to greater than 95 % chain breakage over control DNA (Lindahl and Andersson, 1972). The DNA chain breaks were found at the 3' side of the apurinic sugar moiety between the sugar and and the phosphate residue (Lindahl and Andersson, 1972). The chain breakage was also considered a significant mechanism of heat-induced lethality in bacterial spores (Grecz and Bruszer, 1981). In addition to depurination and hydrolysis of phosphodiester bonds, cytosine deamination was also present, although DNA thermodegradation was inhibited by physiological salt concentrations of 50–1000 mM KCl and 5–10 mM $MgCl_2$ (Marguet and Forterre, 1994). However, abasic sites were spontaneously generated under physiological conditions by hydrolysis of the *N*-glycosylic bond (Kubo *et al.*, 1992). More recently it was found that covalently closed circular plasmid DNA can initially withstand denaturation at temperatures of 95–107 °C, but subsequent depurination events eventually lead to DNA strand breakage followed by denaturation (Masters *et al.*, 1998).

Spores generally have heat resistance mechanisms that extend heat tolerances

above vegetative cells. DNA repair mechanisms have been shown to have a significant influence on heat resistance (Hanlin *et al.*, 1985). Heat resistance of spores is attributed in part to restricting the mobility of heat-labile components of the spore core such as proteins and DNA (Gombas, 1983). Previously, it was assumed that increases in sporulation temperature increased heat resistance as a result of spore mineralization and dehydration stabilizing internal spore components (Beaman and Gerhardt, 1986; Palop *et al.*, 1999). Dehydration of the spore core has been recently shown to measurably alter spore components, resulting in the increased resistance to heat (Novak *et al.*, 2003). Melting of spore DNA may occur at temperatures above 90 °C and is reflected in differential calorimetric scans of spores as an endothermic peak at 90–91 °C (Belliveau *et al.*, 1992; Teixeira *et al.*, 1997). In contrast, spore DNA is further protected by small acid soluble proteins (SASPs) that bind tightly and specifically to the A form of DNA, reducing the rate of depurination *in vitro* by at least 20-fold (Fairhead *et al.*, 1993; Lindahl, 1993; Setlow, 1994, 1995). The SASPs are also effective protectants against DNA damage from dessication, oxidation and UV irradiation (Setlow, 1995). The SASPs protect DNA so well against damage that cell death may be correlated with damage of molecules other than DNA (Setlow, 1995). A SASP-like protein in *Clostridium perfringens* has been observed to cross-react immunologically with antiserum raised against *Bacillus subtilis* SspC (Novak *et al.*, 2001).

Additional evidence in support of the role of DNA in thermoresistance was found in an observed correlation between spore DNA content and elevated heat resistance. (Belliveau *et al.*, 1990). Dipicolinic acid (DPA) or calcium-DPA complexes with DNA were also correlated with spore heat resistance (Lindsay and Murrell, 1985). Whatever the DNA protective mechanism involved, DNA stability is vital to thermoresistance in a microorganism.

An increasing amount of evidence suggests that ribosome damage and degradation is the cause of cell death following thermal stress (Lee and Goepfert, 1975; McCoy and Ordal, 1979). Ribosome denaturation occurs in the same temperature region as thermal inactivation. Numerous investigators have used differential scanning calorimetry (DSC) to examine thermal transitions as indicators of potential sites of cellular injury. (Anderson *et al.*, 1991; Teixeira *et al.*, 1997; Novak *et al.*, 2001). The DSC is an effective technique in measuring changes in protein denaturation temperatures with corresponding changes in denaturation enthalpy. (Kijowski and Mast, 1988). It was suggested that half the enthalpy of ribosome denaturation is associated with protein denaturation (Mackey *et al.*, 1991). Allwood and Russell (1967) observed a direct correlation between loss of RNA and heat-induced loss of viability of *Staphylococcus aureus* at temperatures up to 50 °C. Magnesium is known to have a stabilizing effect on ribosomes. In a study involving mild heating of *Staph. aureus*, Hoa *et al.* (1980) reported that heating results in membrane damage, leading to the loss of Mg^{2+} ions and destabilization of the ribosomes. Depletion of Mg^{2+} leads to 70S ribosome dissociation into 30S and 50S subunits, ribonuclease inactivation, and destruction of 30S subunits (Hurst, 1984; Hurst and Hughes, 1978, 1981). Earlier studies including a number of bacterial species showed that the 30S ribosomal subunit is

specifically destroyed during heat treatment, while the 50S ribosomal subunit appears to be stable, and that 16S rRNA is the prime target of degradation in the heat-injured cells, while 23S rRNA appears to be unaffected (Rosenthal and Iandolo, 1970). Miller and Ordal (1972) examined the rRNA profiles of cells at various times during heat injury at 47 °C. The degradation of rRNA and ribosomal subunits occurs differently during heat injury; the 16S and 30S subunits are affected more readily following heating for 5 min, and the 23S and 50S subunits are degraded more slowly, disappearing after 30 min of heat treatment.

Stephens and Jones (1993) proposed that the protection of the 30S subunit is a critical mechanism for increased thermotolerance. In their study, the osmotic and heat shock-induced increased thermotolerance response of *L. monocytogenes* was concurrent with increased thermal stability of the 30S ribosomal subunit, as measured by DSC. The authors proposed that the stabilization of the subunits occurred through cellular dehydration, leading to an increase in the internal solute concentration, including Mg^{2+} ions, which may contribute to tighter coupled particles of the 30S subunits. Tolker-Nielsen and Molin (1996) reported that heat lethality of *Salmonella* Typhimurium coincides with a significant reduction in the cellular content of 16S ribosomal RNA, thereby suggesting that the degradation of ribosomal RNA is a direct cause of cell death. This conclusion is based on the findings of carbon-starved and magnesium-supplemented cells, which survive heat treatment much better and which also maintain stable levels of ribosomal RNA.

Proteins and enzymes are also considered to be potential sites responsible for heat lethality. It has been postulated that water that is in close contact with the proteins inside the cell could be a factor determining the cell's inactivation. As the cell is heated, water molecules begin to vibrate, and this vibration causes the disulfide and hydrogen bonds in the surrounding proteins to weaken and break, altering the final three-dimensional configuration and possibly preventing the protein from functioning (Earnshaw *et al.*, 1995). The crucial protein that is the rate-limiting, primary target in heat killing is unknown, but the current belief is that membrane proteins may be denatured by heat initially because of peripheral locations followed by the denaturation of crucial proteins within ribosomes (Belliveau *et al.*, 1992). There is evidence that catalase and SOD may be sensitive to heating. These enzymes detoxify oxygen radicals like superoxide and hydrogen peroxide, which form spontaneously in the presence of oxygen and, if undisturbed, can result in death of cells as a result of lipid peroxidation and membrane damage (Kellogg and Fridovich, 1975). A recent study identified a heat-induced 22 kDa protein, rubrerythrin, from *Cl. perfringens*, that may play a role as a scavenger of oxygen radicals under stressful conditions (Novak *et al.*, 2001). Warth (1980) observed a range of sensitivities for spore enzymes and concluded that the enzymes in extracts of spores were inactivated at temperatures ranging from 24 to 46 °C lower than those needed to inactivate the same enzymes within intact spores. Membrane-bound ATPase has been associated with heat resistance/sensitivity of microorganisms. Coote *et al.* (1991) suggested that ATPases are essential for the basal heat resistance of the cell to cope with elevated temperatures. Nonetheless,

thermotolerance induced by sublethal heating is a mechanism independent of ATPase activity.

Flowers and Adams (1976) suggested that the cell membrane is the site of thermal injury of spores subjected to mild or sublethal heating; membrane damage consequently increases sensitivity to environmental stresses. When spores are lethally heated, damage to the membrane permeability barrier results in the release of intracellular constituents and there is a temperature-dependent progressive loss of calcium and dipicolinic acid (DPA) (Brown and Melling, 1973; Hunnell and Ordal, 1961; Rode and Foster, 1960). The death of spores proceeds faster than the release of DPA (Belliveau *et al.*, 1992). When vegetative cells are heated, there is a rapid efflux of ions, amino acids, and low molecular weight nucleic acid components, thereby suggesting that interference with the semipermeability of membranes is a common consequence of heating (Tomlins and Ordal, 1976).

16.5 Strategies to counter pathogen resistance

For strategies to effectively control growth and survival of microorganisms, they must overcome homeostatic mechanisms that the microorganisms have evolved to resist stress (Gould *et al.*, 1995). Food handling conditions should be optimized for maximum microbial lethality during cooking. For example, storage of foods at low temperatures may affect the response of pathogens to subsequent stresses; *E. coli* O157:H7 is resistant to freezing in ground beef (Pandhye and Doyle, 1992) and chicken meat (Conner and Hall, 1996).

Additionally, the heat resistance of *E. coli* O157:H7 in a nutrient medium and in ground beef patties was reported to be influenced by storage and holding temperatures (Jackson *et al.*, 1996). Cultures stored frozen (–18 °C) had greater heat resistance than those stored under refrigeration (3 °C) or at 15 °C, perhaps due to physiological changes within the bacterial cell as a result of freezing (Jackson *et al.*, 1996). Another study (Katsui *et al.*, 1982) showed that the exposure of *E. coli* to 0 °C before heating significantly increased the heat sensitivity of the exposed cells. Juneja *et al.* (1997) reported that the heat resistance of *E. coli* O157:H7 inoculated in ground beef was not altered after storage at 4 °C for 48 h. It is generally accepted that the heat shock response and exhibition of increased thermotolerance is rapidly lost upon chilling and rewarming of cells. Arguably, Williams and Ingham (1997) refuted the hypothesis that short-term temperature abuse significantly increased the heat resistance of *E. coli* O157:H7 in ground beef.

Growth of microorganisms is generally inhibited at pressures in the range of 20 –130 MPa, whereas higher pressures between 130 and 800 MPa could result in cell death (Abee and Wouters, 1999). Both high pressure and high temperature destabilize the quarternary structure of proteins (Jaenicke, 1981). An effective strategy for control of foodborne pathogens may include high hydrostatic pressures in combination with heat treatments. It is presumed that an increased proportion of dissociated ribosomal subunits as a result of high-pressure treatment could induce a sigma-32 factor-dependent heat-shock response (Craig and Gross,

1991). Alternatively, high pressure may affect the phosphorylation state or AT-Pase activity of the heat shock protein, DnaK, which in turn could also modulate the heat shock response (McCarty and Walker, 1991).

The food industry requires a better understanding of the kinetics and mechanism of pressure inactivation before adoption of pressure-based preservation processes. *E. coli* can acquire high levels of resistance to pressure killing by spontaneous mutation (Hengge-Aronis, 1993). The authors used alternating cycles of exposure to high pressure and outgrowth of surviving populations to select for highly pressure-resistant mutants of *E. coli* MG1655. Three barotolerant mutants (LMM1010, LMM1020, and LMM1030) were isolated independently by using outgrowth temperatures of 30, 37 and 42 °C. Survival of these mutants after pressure treatment for 15 min at ambient temperature was 40–85 % at 220 MPa and 0.5–1.5 % at 800 MPa, while survival of the parent strain decreased from 15 % at 220 MPa to 2×10^{-8} % at 700 MPa. Two of the three mutants (LMM1020 and LMM1030) also exhibited higher heat resistance, expressed as increased *D*-values at 58 and 60 °C, and lower *z*-values than those for the parent strain. Interestingly, the ability of the mutants to grow at moderately elevated pressure (50 MPa) was reduced at temperatures above 37 °C, suggesting that resistance to pressure inactivation in these mutants is unrelated to barotolerant growth. The generation of increased pressure-resistant mutants questions the safety of high-pressure food processing, and may have significant implications for the successful application of high-pressure processing in food preservation. Spore-formers are known to exhibit enhanced pressure resistance as well; therefore, it is recommended that high-pressure technologies be used in combination with other treatments to be truly effective (Bower and Daeschel, 1999). Significant reductions in *Bacillus anthracis* spores were recently obtained using 500 MPa pressure and held at 75 °C (Clery-Barraud *et al.*, 2004).

Jorgensen *et al.* (1995) used the submerged coil heating apparatus to determine the effect of osmotic up-shock and down-shock, and osmotic adaptation using different levels of NaCl on the corresponding changes in thermotolerance of *L. monocytogenes*. Subjecting cells to an osmotic down-shift (1.5 to 0.09 moles/ml) caused a rapid loss of thermotolerance, rendering cells 10-fold more heat sensitive than cells grown and heated in TPB containing 1.5 moles/ml NaCl. Subjecting cells grown in media containing 0.9 moles/ml NaCl to a short osmotic up-shock in media containing 0.5, 1.0 or 1.5 mol/ml NaCl resulted in an 1.3, 2.5 and 8-fold increase in thermotolerance, respectively. When cells were adapted to high salinities, an additional two- to threefold increase in thermotolerance occurred compared with cells subjected to an osmotic up-shock at the equivalent level of NaCl. Thus, varying degrees of physical dehydration would lead to enhanced thermotolerance of the foodborne pathogen. The increased thermotolerance observed during the extended exposure to high salinities might be associated with the degree to which the cells have undergone deplasmolysis and accumulated compatible solutes, i.e. the concentration and composition of intracellular solutes. According to Piper (1993), increased thermotolerance could be a result of increased structurization of the intracellular water. This mechanism could be linked with the enhanced

thermostability of ribosomal contents known to occur by both osmotic dehydration and heat shock in *L. monocytogenes* (Stephen and Jones, 1993).

Survival of *L. monocytogenes* in low-temperature environments and high salt concentrations is attributed to the accumulation of the osmoprotectants glycine betaine and carnitine (Sleator *et al.*, 2001). Low-temperature growth requires, in addition to membrane fluidity, mechanisms for regulating the uptake or synthesis of solutes and the maintenance of macromolecular structural integrity of ribosomes and other components important for gene expression and metabolism (Wouters *et al.*, 2000). Proteins (7 kDa) produced in response to temperature downshock or a sudden decrease in temperature are known as cold-shock proteins (CSPs).

In a study by Miller and Eblen (1997), the submerged coil heating apparatus was used to determine whether *L. monocytogenes* cells are more vulnerable to heating after a cold shock. In the model system, cultures were cold-shocked by a temperature down-shift from 37 °C to 15 °C or 0 °C for 0, 1 and 3 h. Cold-shocked and control samples were then evaluated for thermal resistance at 60 °C. The results indicated that the cells grown at 37 °C to stationary phase, cold-shocked at 0 °C for 3 h, then heated at 60 °C, exhibited lower *D*-values than control cells that were not cold shocked. The decrease in *D*-values at 60 °C ranged from 25 to 40 % for two *L. monocytogenes* strains and a strain of *L. innocua*.

In a second experimental series by Miller and Eblen (1997), the effect of cold shock on thermal resistance (D_{60}-values) of cells grown at 37 °C to either lag, exponential or stationary phase was determined. Stationary cells were over 50 % more thermally resistant (D_{60} = 1.27 min), than lag and exponential cells, which had D_{60}-values of 0.83 and 0.79, respectively. When these cells were cold-shocked at 15 °C or 0 °C prior to heating at 60 °C, *D*-values were lowered by 42 %, 30 % and 8 % compared with non-shocked controls for stationary, lag and exponential cells, respectively. The authors pointed out that the maximum effect was in stationary-phase cells, which would most likely be expected to be present in contaminated foods.

Some physical preservation treatments may be best when applied in combination with other technologies. Ultraviolet irradiation (254 nm) can cause cumulative damage to microbial DNA (Bower and Daeschel, 1999). Sublethal UV irradiation leads to the induction of numerous proteins as well as increased protection against heat (Duwat *et al.*, 2000). Although the methodology is effective in decreasing cell numbers, it does not result in complete sterilization and, therefore, cannot be recommended as a definitive process to sanitize foods by itself (Bower and Daeschel, 1999).

Ionizing radiation is known to damage microbial DNA. At temperatures above freezing, cellular inactivation by DNA disruption and production of hydroxyl radicals occurs (Buchanan *et al.*, 1999). At freezing temperatures, DNA damage was the cause of irradiation inactivation and not cellular membrane disruption (Kim and Thayer, 1996). Detrimental effects of ionizing radiation on food products include oxidative rancidity of lipids, which can be prevented by vacuum packaging, and a loss of some minor vitamin components (Farkas, 1987).

Although bacterial spores are more resistant, synergistic effects of gamma

irradiation and heat may be used to control spore-formers since the heat sensitization of irradiated spores is not readily repaired (Gombas and Gomez, 1978). Unfortunately, as with other food preservation methods, there is some indication of an acid (low pH)-induced cross-protection against gamma radiation sensitivity in enterohaemorrhagic *E. coli* (Buchanan *et al.*, 1999). It is important to be mindful that the limitations of radiation that may be applied to a particular product are also determined by the organoleptic changes that occur (Grant and Patterson, 1991).

16.6 Future trends

The use of heat for the inactivation of microorganisms is the most common process in food preservation today. Heat treatment designed to achieve a specific lethality for foodborne pathogens is one of the fundamentally important strategies used to ensure the microbiological safety of thermally processed foods. Heat resistance of microorganisms can vary depending on the species and strain of bacteria, food composition, physiological stage of microbial cells or spores, and recovery conditions (type of media, temperature, atmosphere, and time of incubation) for the detection of survivors. Food characteristics leading to increased thermal resistance of an organism include water activity and the presence of carbohydrates, lipids, proteins, salt, etc. Heat resistance of spores is attributed primarily to thermal adaptation, mineralization and dehydration. Alterations in membrane fatty acid profile results in an altered response to subsequent heat treatment. Potential targets of heat damage include nucleic acids, proteins and cellular membranes.

Quantitative knowledge of the factors in food systems that interact and influence the inactivation kinetics are required to accurately estimate how a particular pathogen is likely to behave in a specific food. There is a need for a better understanding of how the interactions among preservation variables can be used for predicting the safety of minimally processed, ready-to-eat foods. The effects and interactions of temperature, pH, sodium chloride content and sodium pyrophosphate concentration are among the variables that researchers have considered when attempting to assess the heat-inactivation kinetics of foodborne pathogens. Incorporation of these multiple barriers increased the sensitivity of cells/spores to heat, thereby reducing heat requirements and ensuring the safety of ready-to-eat food products.

The future of thermal death determination of bacteria will probably rely on predictive thermal inactivation kinetics modeling. Complex multifactorial experiments and analyses to quantify the effects and interactions of additional intrinsic and extrinsic factors and development of 'enhanced' predictive models are warranted to ensure the microbiological safety of thermally processed foods. In view of the continued interest in minimally processed foods, it would be logical to define a specific lethality at low temperatures. It would be useful to determine the possible effects of injury to vegetative cells and spores that may result from mild heat treatments and the factors in foods that influence the recovery of cells and or spores heated at these low temperatures. In conclusion, future research should focus on

conducting dynamic pasteurization (low-temperature, long cooking-time) studies to assess the integrated lethality of cooking and develop integrated predictive models for pathogens for the thermal inactivation, injury, repair and behavior in ready-to-eat meats, including those packaged in modified atmospheres.

16.7 Sources of further information and advice

Information on expected growth characteristics of foodborne pathogens with respect to food environment variables and holding temperatures can be obtained using the US Department of Agriculture's (USDA) pathogen modeling program, accessed at http://www.arserrc.gov/mfs/pmparameters.htm. Recommended safe cooking temperatures can be accessed from the Illinois Department of Public Health at http://www.idph.state.il.us/about/fdd/safecooktemp.htm. The *Bad Bug Book* brings together facts regarding food, foodborne pathogenic microorganisms and natural toxins from the Centers for Disease Control and Prevention (CDC), the USDA Food Safety Inspection Service (FSIS), the National Institutes of Health (NIH) and the Food and Drug Administration (FDA) at http://vm.cfsan.fda.gov/mow/intro.html. The USDA Economic Research Service (ERS) foodborne illness economic cost calculator can be accessed at http://www.ers.usda.gov/data/foodborneillness. Disease facts from the CDC's Morbidity and Mortality Weekly Report can be accessed at http://www.cdc.gov/MMWR. The responsibility for heat inactivation of foodborne pathogens in foods ultimately lies with the educated consumer. Adequate guidelines are available for the proper cooking, cooling and storage of food products to enable the avoidance of most cases of foodborne illness today.

16.8 References

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